THE THERMOPHILIC MICROÖRGANISMS

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The phenomenon of thermobiosis, including both survival and growth at elevated temperatures, has attracted the attention of biologists sporadically for almost two centuries. The earliest recorded observation was that of Sonnerat (246) in 1774 in which fish were described living at a temperature of 69 R. (*ca.* 82 C) in a thermal pool on the Island of Luzon. This observation received a published letter of confirmation from the Commissioner of the French Navy (206). Fifty years elapsed before a revived interest revealed algae growing in thermal springs (*cf.* 284). Soon fishes, molluscs, arthropods, worms (76, 134), molds (160, 201, 97), and members of all the classes of algae (240, 51, 107, 161, 203, 84, 26, 76, 45, 242) were found growing at temperatures of 60 to 98 C. The extensive literature before 1860 was of a purely descriptive nature without intent of explaining such a peculiar phenomenon (190).

With the development of the field of bacteriology came the description of many more fungal species from thermal springs. Although the work continued in a descriptive vein, observations were presented more completely, and theories of the origin and nature of these unique organisms became numerous.

Although the initial discovery of a thermophilic bacterium is attributed generally to Miquel (175) in 1879, the early descriptions of Long (134), Hooker (117), and Brewer (26) indicate that among their thermophilic "Confervae" were representatives of the *Chlamydobacteriales*. A complete bibliography of early workers (prior to 1927) who have described thermophilic rods, both sporulating and non-sporulating, is given by Robertson (225). A study of the papers cited in this source and the great number of publications in the last twenty-five years dealing with the spoilage of food, particularly processed foods, by thermophilic microörganisms indicates that the bacilli are the forms most commonly encountered. Among the cocci, we find sarcinae (2, 99), staphylococci (51, 232, 257), and streptococci (264, 98, 199) described as thermophilic, although it is more likely that their capacity was one of resistance rather than active growth at elevated temperatures. Specific reference to the higher bacteria has been made largely to the actinomyces (97, 139, 258, 259, 178, 179, 234, 261, 96, 29, 171, 172, 238, 208, 19, 146, 126, 173, 235). Two thermophilic spirochaetes

(41, 28) have also been noted. Higher fungi of thermophilic character have been described (259, 238, 149, 171) and a summary has been presented by Noack (188).

The ubiquitous nature of the thermophiles is attested to by the great variety of sources from which they have been isolated—from freshly fallen snow (99, 215), to the sands of the Sahara Desert (187). They have been found to occur in the air (175, 234), the soil of temperate (97, 163, 215, 193, 139, 96, 23, 237, 258, 259, 234) and tropical (150, 97) regions, salt (163) and fresh water, both cold (175, 215, 193, 170, 44, 257) and thermal (45, 163, 137, 201, 178, 179, 79, 93, 94, 242, 260, 18); on grains and foods of all varieties (264, 215, 193, 154, 205, 13, 236, 133), in raw and pasteurized milk (215, 234, 156, 83, 275, 232, 29); in the feces of all domestic animals and man (175, 163, 215, 258, 259, 234, 261, 29, 8, 69), birds and a variety of fishes and frogs (215); and in stored vegetable material (171, 163, 164, 24, 13, 238, 133, 14, 266, 33, 103). Older reviews may be consulted for the detailed account of the occurrence of these organisms in water (8, 181), soil (149, 150, 171, 146), milk (250, 225, 208, 135), and food (2, 39, 165).

Despite the fact that climatic conditions apparently have no influence on the distribution of thermophilic bacteria (176), several instances of soils free from thermophiles are of note. Migula (174) states that uncultivated soils may be entirely free from thermophiles, and Tsiklinsky (262) was unable to find thermophilic microörganisms in the samples of soil collected from antarctic regions by the Polar Expedition of Charcot. Nevertheless, there seems little doubt that the thermophilic bacteria are natural soil inhabitants and can be isolated from any material which has come in contact with soil.

DEFINITION OF TERMS AND CHARACTERISTICS OF THERMOPHILIC MICROÖRGANISMS

Each worker who isolated one of the so-called "thermophiles" gave it a species designation or number, but failed to provide sufficient data to make possible a comparison among organisms. Even today this vague group of bacteria termed "thermophiles" has few adequately defined species.

Prior to the discovery of thermophilic bacteria, mention was made only of the ability of an organism to live at high temperatures. When bacteria were found with this same thermal tolerance, an effort was made to define the temperature range for growth. Although this range had considerable latitude, any bacterium which grew at elevated temperatures was considered a "thermophile", regardless of its optimum or minimum temperatures for growth. The maximum temperature tolerated generally lay between 50 and 90 C; and the optimum range for the apparent maximum population yield was slightly below the maximum temperature for growth (2). Occasional reference was made to "strict" or "obligate" thermophiles which failed to grow at lower temperatures.

In 1898 Schillinger (237) made great point of the unsatisfactory condition of the literature on the thermophiles and stressed a change in nomenclature. He

proposed the term "thermotolerant" for bacteria which grew at both high and low temperatures and reserved the term "thermophilic" for bacteria which grew at high temperatures but not at body temperature, although he questioned the existence of the latter group. Sames (234) also stressed the importance of this distinction.

By 1907 Miehe (171) had completed his most extensive comparative study of all available cultures of thermophilic species. The purpose was not only to establish synonymity among the members of this group, but also to make a comparison of the general properties of this group and other bacteria. Although Miehe points out a definite compactness of the thermophiles as a group, he cannot deny the difficulty involved in fixing the limits of the temperature ranges for the various groups of bacteria. He arrived at the following nomenclature. *Thermophiles:* organisms with a temperature minimum of about room temperature (25 C); *Orthothermophiles:* thermophiles with a temperature maximum above the temperature of protein coagulation (60–70 C); *Thermotolerants:* organisms with a temperature maximum of 50–55 C, but which also grew well at room temperature.

Little use was made of the criteria set up by Miehe, and Schillinger's designations were employed more frequently. Bergey (19) divides thermophiles into two groups: *True Thermophiles*, which show optimum growth at 60–70 C and no growth, or only very feeble growth, below 40 to 45 C; *Facultative thermophiles*, which develop at room temperature, about 20 C, and have their optimum temperature at about 50 C, and their maximum at about 60 C.

Morrison and Tanner (182) believe that the difference in temperature ranges for the various groups of bacteria can be expressed best by a system of classification based upon the optimum temperature for growth, rather than the temperature limits for growth. Thus, they believed that the organisms that grew at elevated temperatures could be placed in a "homogeneous" group, consisting of the following types: *Strict Thermophiles*, optimum temperature above 55 C; *Facultative Thermophiles*, optimum temperature 50–55 C; *Thermotolerant Bacteria*, optimum temperature 40–50 C. Cameron and Esty (38) have also used the term *facultative thermophile*, but without reference to optimum temperature, the interest being merely in distinguishing between *obligate thermophiles* (grow at 55, but not at 37 C) and *facultative thermophiles* (grow at 55 and 37 C). These terms have since been used commonly in describing organisms isolated from milk (e.g., 71, 208). It was found necessary also to coin new words to describe the heat-resistant flora of milk, e.g., *thermoduric mesophiles* (71, 225–8) which exhibit optimum growth between 20 and 37 C and survive pasteurization in large numbers. Molds generally have been considered thermophilic if their optimum temperature for growth is between 40 and 50 C (188).

The most recent designations, reviving the early descriptive terms of Fischer (82) and the animal physiologists, are those of Imsenecki and Solnzeva (129). "True thermophiles" are those species whose optimal range of growth lies between 55 and 60 C. They may be divided into two groups: "Stenothermal thermophiles", develop at 60 C but show no growth after many days at 28–30

C; "Eurithermal thermophiles", develop at 60 C, and show slight to abundant growth at 28–30 C.

The multiple connotations of the terms employed to describe thermophiles has led to much misinterpretation. The significance of such descriptive terms in a classification of thermophiles is questionable. As in other taxonomic studies, we must concede the impossibility of dividing the thermophiles arbitrarily into a number of groups with specific temperature ranges. From a survey of past work, it would seem adequate to designate bacteria with an optimum temperature for growth between 50 and 60 C as thermophiles, and, if necessary, to use the terms of Imsenecki and Solnzeva for defining the magnitude of the temperature range for growth.

The question of synonymity of species names was apparent early in the study of thermophilic bacteria. Inadequate description of the characteristics of an organism, coupled with failure to maintain type cultures for comparative purposes, has led to a confusion of duplicate names. Bruini (29), Miehe (171), Morrison and Tanner (181), and Prickett (208) have contested the identity of many of the species presented in the literature. However, on the philosophy that less confusion is caused by an organism having two names than by two organisms having the same name, new names have continued to appear in the literature. For example, Beaver (11) has described 32 new species of thermophilic spore-forming rods with very descriptive species names. Species distinctions were based upon such insignificant differences as the relative time required to effect a particular biochemical change, the abundance of growth on a particular medium, and so on. The strikingly similar morphology and physiology of the aerobic and facultative thermophiles (38, 181, 182) has led several workers to the assumption that all the spore-forming organisms are variants of a few type species.

The Committee on Classification of the Society of American Bacteriologists under Bergey (20) has attempted to standardize the terminology of this group, and the subsequent revision by Chester (21) presents descriptions of 21 species of the Thermophilic Group, Family *Bacillaceae*. The scheme is far from satisfactory, but is serviceable in that it presents in convenient form a portion of our knowledge of this type of organism. The problem of segregating the assorted information in the literature is discussed by Prickett (208). Our present classification of thermophilic bacteria, based upon slight differences in spore size and location, ephemeral pigments, etc., of inadequately studied cultures, assists very little in the identification of a newly isolated organism.

Common morphological characteristics of aerobic and facultative spore-forming thermophiles, described up to 1928, have been pointed out in detail by Prickett (208). In addition to the similarities cited, all these thermophiles, in youth, are gram positive, with the possible exception of *Bacillus stearothermophilus* (66). It is interesting to note that of all aerobic and facultative spore-forming organisms of this group there is only one species listed which produces gas in its utilization of carbohydrate materials. However, the original, and only, description of this organism, *Bacillus thermoamylolyticus* (52), indicates that the investigator was working with a mixed culture.

Equally interesting is the observation that the literature reveals few cellulolytic thermophiles with adequate description for comparative purposes. Despite the early interest of MacFadyen and Blaxall (164) and the detailed studies by Omelianski (191), Pringsheim (211-3), Kroulik (147), Tetrault (254) and others of the University of Wisconsin group (*cf.* 186, 189), and the recent Russian workers, little knowledge of the thermophilic cellulose-digesting organisms in pure culture has been gained.

Superficial studies and ill-defined species have resulted from the industrial interest in the use of these organisms for the disposal of cellulose wastes. However, the tremendous difficulty in establishing unequivocally the purity of cultures of thermophilic cellulose bacteria is responsible largely for the confusion in this group of thermophiles. The purity of aerobic and facultative cultures capable of hydrolyzing cellulose (268, 241, 140, 202, 245, 12, 147, 53, 283, 254, 255, 87, 132) long was questioned (214) and still is to be contested. The symbiotic nature of most of the cellulose fermentations prevents us from attributing all of the products to thermophilic activity. The wide variety of gases, acids, and neutral volatile materials is illustrated well by the summary of Buswell and Hatfield (34).

Cultures which are active in digesting cellulose when cultivated aerobically and anaerobically have been found frequently to include an anaerobic species and a facultative species. *Clostridium thermocellum*, described by Viljoen *et al.* (268), is thought now to be such an association. It is recognized also that obligate anaerobes (263) are composites of associated forms (244). Murray (185), however, has obtained pure cultures of aerobic and thermophilic cellulose-digesting bacteria. The inability of other workers to obtain aerobic growth in agar of thermophilic cellulose-splitters has been attributed to inadequate humidity. A saturated atmosphere was found to be necessary for optimum growth of these organisms.

The symbiotic nature of cellulose fermentation has been clarified recently by the Russian microbiologists (122, 127, 230, 120, 229, 231, 121). Anaerobic decomposition of cellulose by thermophiles has been resolved into two processes: the hydrolysis of cellulose, and the subsequent fermentation of hydrolytic products. In pure cultures of cellulolytic thermophiles most of the hydrolytic products (40 to 75% of the cellulose carbon as glucose) accumulate in the medium, and only a portion is fermented to yield carbon dioxide, hydrogen, and acetic, butyric, formic, and lactic acids. In mixed cultures, i.e., in symbiosis with other organisms, higher yields of acids and alcohols, and products, such as methane, not formed in pure culture fermentation, are obtained. The highly cellulolytic activity of the thermophiles thus effects an accumulation in the medium of a carbon compound which is readily fermentable by the concomitant organisms. The activity and characteristics of pure cultures of a number of cellulolytic thermophiles, such as *Bacillus cellulosaedissolvens* and *Clostridium illiposporogenes*, have been reported.

By definition, organisms other than the spore-formers also must be grouped with the thermophiles. Most notable of these are several of the lactic acid bacteria. Henneberg (114) and Orla-Jensen (195) have described lactic acid

bacteria with an optimum temperature in the region of 50 C. However, Tsiklinsky (263), one of the outstanding investigators of thermobiosis, a few months before her death in 1921, isolated lactic acid bacteria with elevated optima, and with minima between 42 and 45 C. *Lactobacillus thermophilus* (7, 47) of Orla-Jensen's sub-genus *Thermobacterium* is the only well-studied organism which, has a temperature optimum above 50 C (optimum 50–62.8 C; minimum 30 C; maximum 65 C). Other non-sporulating rods and cocci, which have been considered to be thermophiles, require more study before they can be placed in this category. Cocci which have been called thermophilic (257, 99, 199, 195, 264, 232) undoubtedly multiply at temperatures in excess of 50 C, but find their optimum far below this point. The cocci and non-spore-forming rods presented in the early descriptions of van Tieghem (264), must be discounted. *Denitrobacterium thermophilum* of Ambrož (3) from all indications was a thermophile. There are probably also thermophilic filamentous bacteria with optima above 50 C, but a summary must await a more detailed study of these forms.

Among the anaerobic spore-forming thermophiles we find greater confusion than among the aerobes. The generic name *Clostridium* has been applied indiscriminately to facultative anaerobes (154, 205). Obligate anaerobes (236, 187, 265, 261) and facultative anaerobes (193, 18, 8) have been described briefly on a number of occasions, but given species names infrequently. Confusion with regard to the oxygen requirement of some organisms has arisen as the result of the misconception (215, 238) that aerobic thermophiles with high temperature minima will grow at ordinary temperatures if cultivated anaerobically.

The best defined spore-forming anaerobes of the thermophilic group are *Clostridium nigrificans* of Werkman and Weaver (280, 279) and *Clostridium thermosaccharolyticum* of McClung (166), both with a temperature optimum at 55 C or higher. *Clostridium thermoacidophilum*, *Clostridium thermoaerogenes*, *Clostridium thermochainum*, and *Clostridium thermoputrificum* are obligate anaerobes described at length by Damon and Feirer (61). Werkman (279), however, was unable to confirm the anaerobic nature of *Clostridium thermoputrificum*, and McClung (166) failed to obtain the proper reactions when studying the other available cultures of Damon and Feirer.

Non-sporeforming obligate anaerobes also may have representatives in the thermophilic group, for some grow very actively, if not optimally, at temperatures well above 50 C. Several bacteroides-like organisms, originally isolated by Veillon (265) and later termed *Bacillus thermophilus* γ and β (277, 207), are grouped in Prevot's genus *Ristella*.

Thermophiles, therefore, constitute a very heterogeneous group, if we include all organisms with an optimum temperature for growth above 50 C. Their morphology, and even staining reactions, are varied. Fundamental differences appear in their nutritional requirements and metabolic activities. Most of the thermophiles will grow well on the common culture media; others require special nutrients. The field of essential nutrilites for these organisms has been untouched.

Although most investigators agree that thermophilic organisms are non-pathogenic, several accounts opposing this view are of note. Bruini (29) injected whole cultures of thermophilic organisms into guinea pigs and attributed death of the animals to the toxic products formed by the thermophilic cells. Ascione (6) described a thermophilic streptothrix which apparently produced a hemolytic toxin. The validity of the results of these two workers is not beyond question. Black and Tanner (22) have reviewed the subject of pathogenicity of thermophilic organisms.

The greatest number of the thermophiles are facultative with regard to the oxygen tension under which they are capable of developing. Despite much conflicting evidence in the literature, there are in addition, aerobic and anaerobic forms. The literature indicates that the facultative organisms exhibit widely different oxygen tension requirements for optimum growth, i.e., some grow best under aerobic conditions, while others are favored by an anaerobic environment.

Most of the thermophilic bacteria are capable of producing spores; but the ability and degree of sporulation under a given set of conditions varies among cultures. The great heat resistance of these spores is assumed generally. The body of data on this subject will be discussed in a later section.

The fate of various substrates shows considerable diversity. Various thermophilic organisms are unable to utilize the simple mono-and di-saccharides (278–280), starch (94, 23, 208), and cellulose. Apart from the mere utilization of carbohydrate material, fundamental differences in endproducts are noted when a carbohydrate is fermented. Thermophilic organisms which attack carbohydrates are most important in the spoilage of foods, and consequently have received greatest attention. Research workers of the National Canners Association and the American Can Company have divided these organisms into two groups and have given numerical, rather than species, designation to members of the groups with differences in cultural reactions, i.e., flat sour organisms: aerobic and facultative bacilli characterized by the production of acids (lactic, formic, acetic) but not gas; gas-forming (non-H_2S) anaerobes: anaerobic bacilli which produce acid and large quantities of gases (CO_2 and H_2) from carbohydrates.

For most thermophiles described in the literature it is impossible to determine the action on protein materials. The universal use of gelatin and milk provides very little information. Early workers (215) were led to conclude that the most important characteristic of thermophilic bacteria was their proteolytic activity. Although some thermophiles have been described as highly proteolytic, such as *Bacillus delbrueckii* (12) and several non-cellulolytic bacteria isolated from manure (69), sewage (8), water (181), and milk (83), the activity of these organisms in symbiotic relationships in nature suggests that they are, at most, feebly capable of attacking native proteins (196). This has been found to be true in more recent work with pure cultures of food spoilage organisms, called hydrogen sulfide or "sulfur stinker" organisms (165, 279), and thermophiles from milk (71). Hydrogen sulfide and indole production are the only end-products of organic nitrogen metabolism studied. Indole is apparently pro-

duced by a limited number of thermophilic organisms (181); hydrogen sulfide is not a common product even among the anaerobes. Reduction of nitrate to nitrite is encountered frequently among the thermophiles (21), although reduction of nitrite is infrequently cited (233). *Clostridium thermosaccharolyticum* is unable to reduce nitrates, but can reduce nitrites (166).

The following types of thermophilic microörganisms have been described: nitrogen fixing (210, 149), nitrosifying (40), denitrifying (80, 19, 3, 22, 243, 150, 8), sulfate reducing (77, 247, 248, 75), sulfur and sulfide oxidizing (179, 242, 58), iron (178); proteolytic (193, 150, 19, 80, 243, 196, 22), amylolytic (193, 23, 19, 52, 243, 22, 130, 123, 125, 128), lipolytic (150); and halophilic (28). A number of thermophiles are capable also of oxidizing phenol and various hydrocarbons (73, 74).

ORIGIN AND DISTRIBUTION OF THERMOPHILIC MICROÖRGANISMS

It is understandable that subsequent to the search for members of this apparently abnormal group, efforts were directed toward establishing their origin, an interpretation of their wide distribution and abundance, and the mechanism by which they are so resistant to heat.

Conjectures as to origin have varied from the logical to the fantastic. Thus, workers, like Rabinowitsch (215, 216), Schillinger (237), Tsiklinsky (258–260), Jancke (135), Miehe (171), and Imsenecki and Solnzeva (129) considered them variants of well-known strains of mesophilic bacteria, progressively more completely adapted to higher temperatures up to the final obligate stage; others (18, 163, 29) considered that they had become adapted even more gradually and more reversibly than connoted by the term "variant". Lieske (158) and Kluyver and Baars (143) believe that they are the result of spontaneous adaptation or mutation, occurring in one step. Thermoresistant forms resulting from mutation have been found in various of the lower animals (16), and Ricket *et al.* (224) have reported an hereditary shift in growth optimum from 36–37 C to 41–42 C for a lactic acid bacterium after treatment with KCl.

Weed (276), and later Ambrož (2), suggested that thermophilic microörganisms may be "reminders of the thermophilic flora of earlier geological periods" —perhaps influenced by descriptions of forms like Renault's giant bacteria of the carboniferous age (223). Molisch (180) and Golikowa (99) also maintain that these organisms have arisen, not by adaptation from lower to higher temperatures, but rather that they have arisen at elevated temperatures and some have become adapted very slowly to lower temperatures. Sames (234), de Kruyff (150), and Miehe (171) have indicated the tropics as the locus of the evolution of thermophilic microörganisms.

Svante Arrhenius (5) discounts such an adaptation process on this planet, and considers the natural habitat of the thermophilic bacteria to be the planet Venus, where the average temperature is 47 to 50 C. The organisms or their spores are believed by him to be propelled by the radiation pressure of the sun and to travel from Venus to Earth in a few days.

In all probability the thermophilic microörganisms had their origin in some locality of tropical climate, and are found today in greatest numbers where elevated temperatures prevail. The role of the thermophilic bacteria in the economy of nature proved to be as puzzling as their origin. Temperatures between 50 and 60 C are not uncommon in tropical soils (105) and the activity of thermophilic forms in this environment is of great significance (150, 97). Their distribution in lesser numbers over the entire surface of the earth opened vast fields of study and speculation with regard to their existence in regions where the temperature seldom, if ever, reaches a maximum of 40 C.

The direct heating action of the sun in the temperate zone has been found (234, 97, 98, 188, 146) to be sufficient to permit multiplication of thermophiles in superficial top soil, mud puddles, and fallen vegetation. In addition, the processes of putrefaction and fermentation, effected by mesophilic organisms, provide adequate heat for active germination and growth of thermophiles (163, 29, 99). Attention was directed, therefore, toward spontaneous heating and combustion of hay and manure piles (171, 172, 150, 51). Maximum temperatures reached within piles of vegetation have been reported to lie between 60 and 90 C (171, 238, 133, 168, 116, 92, 69). Noack (188) is of the opinion that the activity of thermophilic microörganisms does not depend upon man's agricultural pursuits, and presents evidence that the temperature attained in a pile of fallen leaves 50 cm high is adequate for the development of thermophilic flora. The subject of spontaneous heating and combustion of hay has been reviewed extensively by Browne (27).

Other workers (99) believe that, generally, the above situations constitute exceptional conditions under which thermophiles can develop; but in the absence of these conditions, the thermophilic microörganisms continue their activity in a symbiotic relation with mesophilic organisms.

Bacteriologists who consider all thermophiles to be thermotolerant in varying degree have presented voluminous evidence (215, 216, 261, 29, 237, 2) that the thermophilic bacteria thrive in the alimentary canal of man and animals. However, the mere presence of thermophilic bacteria in the intestine of warm-blooded animals does not constitute proof that these organisms find here a suitable habitat for growth. The actual number of thermophilic bacteria has been shown to be small in the case of human feces (4, 22), and perhaps largest in the feces of cattle (22). The part played by these organisms in the decomposition of the intestinal contents is unknown, but discounted frequently as unimportant (99).

An interesting observation on the distribution of thermophiles was made by Mischustin (177) in a study which employed the thermophilic bacteria as an indicator of the "cultivatedness of soil". Thermophilic bacteria were found in insignificant numbers in virgin soil, but upon cultivation of such soil, thermophiles were introduced with the manure, and developed rapidly to great numbers. According to this observer, the number of thermophilic bacteria in soil is closely related to the intensity of manuring.

EXPLANATION OF HEAT RESISTANCE AND GROWTH AT ELEVATED TEMPERATURES

Explanations for the ability of thermophilic organisms to carry on normal life processes at elevated temperatures, incompatible with the usual forms of life, must be based upon random findings drawn from many of the biological sciences. From the pioneer work of Miquel (175) to the early twentieth century (257, 99), the belief that thermophiles contained a peculiar type of protoplasm was the extent of speculation. The high resistance of thermal algae was attributed by many workers to the presence of dissolved gases (111) and other constituents of the medium. The relation of thermal sensitivity of microorganisms and the chemical composition of the medium has been reviewed adequately by Bělehrádek (16).

Davis (63) was unique in his statement that thermophilic forms are able to withstand unusually high temperatures because of their "low grade of protoplasmic organization". Attention, however, soon was deflected from the thermophilic to the mesophilic forms and focused upon the bacterial spore, the most commonly recognized example of thermal resistance. von Esmarch (271) maintained the idea that spores are surrounded by a protective coating which insulates them by partially preventing the passage of heat. This assumption was opposed by Lewith (157) and Virtanen (269); the latter, by calculation, estimated that the spore wall would have to be a million times more insulating than air in order to exert any protective effect. Evidence, however, has been presented for a protective coating, either a capsule (104) or a coating of questionable secretory origin (267, 226, 282, 100, 101). Hückel (118) was able to isolate from several species of mesophiles a non-specific protective substance secreted by the cells which, when separated from the culture by filtration, imparted greater heat resistance to less resistant organisms. While it may be conceded readily that certain materials do exert a protective effect on cells subjected to heat, such a protection in the case of actively metabolizing cells is difficult to conceive. It may be noted, however, that many workers today assume a special role for the spore coat in heat resistance (153).

Before the time of Pasteur, Doyère (68) had demonstrated the effect of water on the thermal resistance of rotifers and tardigrades. A correlation of water content and heat resistance has been noted since then to apply to the spores and vegetative cells of many of the lower plants and animals (59, 62, 198, 239, 226). The assumption, however, that vegetative cells have a higher moisture content than spores was disproved by the work of Virtanen and Pulkki (270) in which it was found that no such difference in water content existed. Cramer (54) and Benecke (17) have described a hygroscopic cell wall of carbohydrate and fat-like material capable of retarding diffusion. By comparing the rates of diffusion of a dye into spores and vegetative cells, Benecke provided evidence for his theory and suggested permeability as a controlling factor in thermal resistance. A score of subsequent observations (226) have confirmed the relative impermeability of the spore, and occasional indications (31) have been found that the degree of permeability of the vegetative cell wall to water de-

termines the heat resistance. Robertson (226) concludes, from a study of thermophiles in milk, that changes in the nature of the "cell-wall membrane", and changes involved through acclimatization processes may be instrumental in producing a cell with a low moisture content and consequently a higher thermal resistance. Also with regard to the thermophilic bacteria, Hampil (104) suggests that vegetative cells of these organisms have a lower water content than cells of mesophiles. Apparent differences in water content may be of significance in an analysis of heat resistance, but of equal importance is the increased surface effects at elevated temperatures.

At the time such early hydration theories were proposed, the existence of bound water in biological systems was unknown. And despite the emphasis on water of hydration by Gortner and other workers (102), its physiological significance is even today a matter of heated argument (30). Evidence has been presented to explain the resistance to low temperatures of certain plants, seeds, spores, bacteria, and animals (113) which apparently contain little water. The work has been based on the fact that bound water has a lower freezing point than free water, by virtue of the strong forces binding it. Although it is known that the tenacity with which some substances retain water of hydration is indicated also by the higher temperatures required to remove it, few published attempts have been made to relate this character of bound water to heat tolerance of bacteria (88). A high bound water content, as found in spores, has been interpreted as a protective mechanism against coagulation of cell proteins. The alteration of proteins, or "irreversible protoplasmic changes", constitute a traditional theory of death of organisms at elevated temperatures (49), despite early demonstrations that the coagulation of proteins does not parallel the thermal death point of the organism (151). Conflicting views were held with regard to the factor responsible for preventing or decreasing the rate of protein coagulation. Shaw (206) contended that in thermophiles the higher specific gravity of the protoplasm was responsible, while Williams (281) and others (19) attribute the observed effect to the low mineral or ash content of resistant forms. Very concrete evidence recently has been provided by the elemental analyses of Curran *et al.* (55) in which the calcium content of spores was found to be considerably higher than for corresponding vegetative cells. The authors note that the high calcium content may be related to the ability to bind water and to heat resistance. Thermophilic bacteria have not been considered as yet for bound water studies.

The observation that usually both death of microörganisms and inactivation of enzymes by heat proceed in logarithmic order, i.e., a first order reaction, has led to the frequent association of death by heat and the thermolability of enzymes. Common, also, is the assumption that heat resistant forms possess peculiar enzyme systems. Virtanen (269) proposed that a firmer combination of enzyme and cell protein is responsible for increased thermal resistance. Feirer (80) has claimed that the enzymes, catalase and diastase, of some soil thermophiles are active at temperatures where the enzymes of mesophiles are destroyed (104). Multiplicity of enzymes or a stronger enzyme-protein associ-

ation have been suggested by Rettger and his students (42, 72) to account for increased resistance. The minimum temperature for destruction of catalase, indophenol oxidase, and succino-dehydrogenase, compared statistically with the maximum growth temperature of a bacterium, was claimed by these workers to show good agreement for the mesophiles and thermophiles studied. A high degree of correlation was apparent for mesophilic cultures, but was questionable for thermophilic cultures. Using nine strains of thermophilic bacteria with a weighted mean maximum growth temperature of 76 C, they found the minimum temperature for destruction of indophenol oxidase to be 65 C, of catalase, 67 C, and of succino-dehydrogenase, 59 C. Rahn and Schroeder (222), however, denied the possibility of concluding from the work of Rettger *et al.* that enzymes exhibit such behavior in normal living cells. The use of resting cell preparations is considered faulty technique in that it provides an abnormal static condition, as opposed to the normal dynamic capacity of a growing organism to produce new enzyme molecules to replace those deteriorated. Thus, the results of heat inactivation of enzymes, determined in resting cell preparations, and maximum growth temperature, determined in a complete medium, are not comparable. Rahn and Schroeder (222), using *Bacillus cereus*, tested the data of Rettger by examining a suspension of cells in phosphate buffer for viability and enzyme activity as a function of temperature and time. Invariably, enormous decrease in number of viable cells was accompanied by only slight decrease in activity of catalase and succinic dehydrogenase, the only two enzymes studied.

Rahn (218) attributed death of bacteria by heat to endogenous catabolism, destruction of enzymes, or the inactivation of genes. The logarithmic order of death of bacteria (48, 274) indicates that death must be due to the destruction of a single molecule. Therefore, an explanation of death based upon the heat inactivation of enzymes (131) is untenable. The general observation that the enzymes of bacteria function at temperatures above the maximum temperature for growth was found valid when extended to yeast (221) and bacterial (67) fermentations, to some of the respiratory enzymes of mesophiles (222), and to the complete respiratory system and its enzymic components in thermophilic bacteria (89). The inactivation of one of the heat labile enzymes involved in the very obscure synthetic reactions may satisfy the mathematical considerations of Rahn. However, such an interpretation of the growth mechanism does not provide the only alternative. Multiplicative reproduction, the bacteriologists' usual criterion of life, is not the inevitable sequel to growth. Growth has occasionally been observed to continue after the faculty of division was lost. The inactivation of a single gene essential to the reproductive mechanism, thus producing a sterile mutant (or "lethal mutant", as used by Jordan, 136), is consistent with the logarithmic order of death by heat and is maintained by Rahn (217, 219, 220) as the explanation of "death" of bacteria. Such an explanation of death implies for thermophilic bacteria a heat stable genetic structure, in addition to the possibility of unique enzyme complexes.

Tolerance of high temperatures has often been associated with the nature of

the lipids. An inverse correlation between the melting point of the fat of an animal and the temperature at which the animal lives has been long recognized (115). The analysis by Leathes and Raper (155) and the extensive review by Bělehrádek (15) contain evidence that the protoplasmic and reserve fats and constituent fatty acids of animals and plants living at relatively low temperatures are more fluid, i.e., less saturated, than the fats of animals and plants living at higher temperatures. Leathes and Raper, in an attempt to explain the observed distribution of saturated and unsaturated fatty acids in nature, advanced an hypothesis based upon the usual theory of fat synthesis, namely the formation of long carbon chains with unsaturated linkages and subsequent saturation by reduction. They maintain that the condensation reaction proceeds readily at low temperatures, giving rise to unsaturated fatty acids, but the reduction processes require a higher temperature. Temperature, however, is not the only factor responsible for the saturation of fatty acids. Terroine *et al.* (252, 253) have verified, in part, the assumption of Leathes and Raper. In a study of *Aspergillus niger* and the timothy-grass bacillus over a temperature range of 14 to 38 C, they observed a greater utilization of the potential energy of the medium at higher temperatures and the occurrence of more saturated fatty acids, both total and phosphatide, at higher temperatures. Such data may be interpreted as indicating that the saturation of fatty acids by reduction succeeded the condensation reaction and involved an additional expenditure of energy. Pearson and Raper (200) have also studied the total fatty acids of *Aspergillus niger* and *Rhizopus nigricans* over a narrower temperature range and demonstrated the influence of temperature on the saturation of the fatty acids formed. The assumptions on which the general hypothesis was based are unproved, however, and the data in accord with the hypothesis are very scant. Nevertheless, in the cases studied, it is clear that the temperature at which fats are formed is one factor which influences the degree of saturation of the lipids.

Heilbrunn (112) and Bělehrádek (15) suggested that the melting point of the protoplasmic lipids determine the heat resistance of an organism. Despite the intriguing aspects of this suggestion, the literature is strikingly devoid of experimental data. Gaughran (89, 90), in a preliminary study of *Bacillus subtilis* and a stenothermophilic thermophile, found that for *B. subtilis* the total lipid and its constituent acetone-soluble fat and phospholipid fractions decrease in quantity and degree of unsaturation as the temperature of cultivation is raised above the optimum, while the lipids of the stenothermophilic bacillus are strikingly constant both in quantity and degree of unsaturation. This point will be discussed in the consideration of growth of thermophiles at low temperatures.

Indirect evidence has been made the basis for many theories offered in explanation of thermal resistance and thermal requirement. Experimental difficulties here are numerous, but not of such magnitude as to account for the deficiency of experimental data on this fundamental problem.

It seems unnecessary to assume a unique protoplasm for the thermophiles or to conjecture about the mechanism by which the protoplasm resists "irre-

versible changes". As physical and chemical data on enzymes, proteins, bound water, permeability, surface phenomena, etc., accumulate, we may find differences between thermophiles and mesophiles. "Irreversible changes", occurring in all cells, proceed at a correspondingly greater rate in thermophiles growing at elevated temperatures. Even at their optimum temperature for growth, many thermophilic bacteria show an extremely high death rate. Therefore, it is most probable that growth of thermophiles is not merely passive resistance to the unfavorable effects of high temperature, but rather may be attributed to their tremendous capacity of replacing compounds destroyed by heat. The rate of destruction is not significant, if the rate of replacement is greater. This situation is reflected clearly in the population curves of the thermophilic bacilli. The duration of time for which thermophilic cells can maintain such an intense metabolic process is limited, and consequently their death rate is exceedingly high. The mechanism of such a balance is, of course, open to conjecture. Porter (204) postulated that the thermophilic cell is controlled by a "governor" of some sort which prevents the rate of catabolism from exceeding that of anabolism until the temperature reaches a certain value, at which point the cell dies.

Earlier it has been pointed out that spores of the thermophilic bacilli are considered to be the most heat tolerant of all bacterial spores. The body of data which has led to this conclusion is large, but the diverse and poorly controlled conditions under which most workers studied the thermal resistance of the spores of thermophiles, provides little basis for a comparison with the resistance exhibited by other species (e.g., 23, 169). Eckford (71) and others (22) have pointed out a direct relationship between the maximum temperature for growth of a thermophilic organism and the heat resistance of its spores. An analysis of a more recent controlled experiment (153) has indicated an inexact, but significant, correspondence of maximum growth temperature and thermal resistance of spores. Other factors than those which determine maximum temperature for growth of the organism are believed to be involved in the phenomenon of thermal resistance.

In accord with the above correlation, Eckford (71), Bergey (19), and Esty and Williams (78) have noted that spores of true thermophiles have a greater heat tolerance than spores of thermotolerant organisms. Black and Tanner (22), however, have found that the spores of most thermophiles are not unusually resistant to heat and that a particular strain exhibited the same resistance to heat, whether the organism was isolated directly from nature or "selected" for heat resistance by isolation from sources previously heated or processed. The spores of only two strains, of the many aerobic thermophiles studied, were found to survive 100 C for 24 hours, 115 C for one hour, and 120 C for 25 minutes. Other investigators have noted cases in which spores of thermophiles have survived autoclaving (184).

NATURE OF GROWTH OF THERMOPHILES

1. Growth at Elevated Temperatures. Growth of thermophilic microörganisms has been determined largely by visual observation. Tanner and Wallace (251)

were the first to apply the quantitative growth-curve method to the thermophilic bacteria. They prepared growth curves for three bacilli at 20, 37, and 55 C. The lag phase at 55 C could be decreased greatly by pre-heating the medium and using an inoculum of young cells. At 55 C, they observed most rapid increase in cell numbers and, after the period of active growth, a rapid death; and since cultures often became sterile, it may be inferred that these thermophiles did not sporulate at this temperature. The absence of spores may have been the result of the very low oxygen tension in a liquid medium at an elevated temperature, in accord with the observed relationship of oxygen tension and the capacity to sporulate among the aerobes, as well as facultative and strict anaerobes (42).

Hansen (106) prepared growth curves of a strain of Cameron and Esty's facultative thermophiles Group 80 with the object of obtaining information about the generation time and rate of fermentation. The growth rate was found to increase with increasing temperature to about 55 C, above which the rate decreased. At the point of maximum viable cell number, the 55 C curve fell off much more sharply than the corresponding curve at 37 C. A generation time of 16 minutes was reported at 55 C. In the presence of glucose, and calcium carbonate to neutralize the resulting acid, the maximum viable cell yield (*ca.* 6 x 10^8 per ml) was obtained at 42 C, rather than at 55 C; in addition, the crop decreased at temperatures above and below 42 C, but again became large at 20 C. The viable crop represented only a fraction of the total count. Hansen maintains that thermophilic cultures become sterile when stored at high temperatures if the acids formed from carbon compounds in the medium are not neutralized. Although the yield of viable cells is low at 55 C (*ca.* 10^8), the rate of fermentation is high enough to effect great chemical changes in a short time. Hansen estimates that the fermentative capacity of this thermophile at 55 C is about thirty times as great as that of *Streptococcus lactis* at 20 C.

Imsenecki and Solnzeva (129) and Gaughran (89) have been unable to demonstrate a lag with a number of thermophilic bacilli, when using inocula consisting of cells from 12- and 17-hour cultures, apparently in the maximum stationary phase of the cultural cycle. In accord with the data of previous workers, growth was characterized by high reproduction and death rates. The logarithmic growth phase, during which the rate of multiplication remains constant, is probably of very short duration and is not evident in the population curves. The total population curve and the viable population curve rapidly diverge to a point at which the total count is 50 to 100 per cent larger than the viable count. Such a divergence is indicative of a rapid death rate. A pronounced negative slope of the latter portion of the total population curve has invariably been noticed. It is first apparent at approximately the same time at which the number of viable cells begins to decrease. Such a decrease in the total number of cells has been attributed to a cytolysis induced by autolysis or an accumulation of toxic products in the medium.

The above observations of cytolysis, augmented by the occurrence of many

"ghost" forms, not included in the total microscopic count, leads to an entirely different interpretation of the population cycle. Additional evidence of this autolysis has been found by Imsenecki and Solnzeva (129) in the very rapid accumulation of enzymes in the medium containing thermophilic organisms. Thus, the generation time of the thermophilic bacilli is very short, perhaps of the order of magnitude of 5 to 15 minutes, and not several hours as indicated by the curves determined in the usual way. The curves obtained represent only a quantitative expression of the numbers of living and dead cells present at any one time in the culture, and gives no indication of the rate of reproduction. Autolysis is present early in the culture cycle and becomes a predominant factor in the latter portion of the population curve. Thus, autolysis effects a lowering of the total population curve and depresses the apparent death rate.

Cultures of stenothermophiles, as a result of a high death rate and autolytic rate, never reach the maximum viable or total populations so common in most mesophilic cultures. In the aerobic cultures the oxygen demand of the rapidly metabolizing cells can not be supplied adequately even in a very shallow layer of medium. The ability of aeration to increase viable cell yield seems to bear some relation to the temperature range for growth of the organism, for thermophiles with a broad temperature range for growth respond to a greater degree than thermophiles with a narrow temperature range.

Imsenecki (123) has found that proteolysis, denitrification, and hydrolysis of starch by thermophiles proceeds at a rate seven to fourteen times that of cultures of mesophilic bacteria. A study of culture populations and the rate of biochemical activity indicate that the high reproductive rate is inadequate in explaining the intense biochemical activities of the thermophilic bacteria. In the case of proteolytic thermophiles in suitable media, the number of viable cells rapidly increases (to *ca.* 80×10^6) and then decreases according to the typical population curve discussed above, while the proteolytic activity gradually increases and reaches a maximum at a time corresponding to the lowest portion of the viable population curve (*ca.* 10×10^6). A mesophile, *Bacillus mesentericus*, on the other hand, shows the usual increase in protein digestion with increase in the number of viable cells. The progress of proteolysis may, of course, be related to the rapid reproduction, death, and autolysis, and a resultant accumulation of proteolytic enzymes in the medium. The behavior of amylolytic thermophiles, however, according to Imsenecki (123), does not present a corroborative picture. Here diastatic activity proceeds at a rate far out of proportion to the number of viable cells in the culture and increases very rapidly during that period in which autolysis is not a predominant factor in the culture cycle. Thus Imsenecki is led to the conclusion that an explanation of the high biochemical activity of thermophilic bacteria is to be found in the very intense metabolic activity of these organisms, and not merely in their rapid proliferation. Russian workers have conducted a number of investigations of the amylolytic (123–5, 128, 130), cellulolytic (121, 122, 127, 229–231), and proteolytic (123) thermophilic bacteria with an appreciation of the potential value of these organisms in industrial application.

The unfavorable effects of the high temperature at which thermophilic bacteria have their designated optimum have frequently been pointed out. Suggestions have been made that, perhaps, the optimum temperature of these so-called thermophiles should be considered as much lower. A number of thermophilic strains have been found to die out if continuously cultivated or even stored at elevated temperatures. The inability to form spores has usually been held responsible in the case of facultative thermophiles. Thermophiles with a narrow temperature range, however, do produce spores at their optimum, but experience a depression in this activity as the maximum temperature is reached. Thus, it is possible that rapidly metabolizing vegetative cells in a depleted medium and a high concentration of a toxic metabolic product, such as acid, would be unable to survive for long, if the cells were unable to produce some resistant form. Experience has shown, however, that in the absence of detrimental metabolic products, thermophilic cultures can be stored indefinitely at temperatures of 50 to 60 C.

Considerably less uniformity in size and proportion has been noted in thermophilic cells cultivated at their optimum temperature than when cultivated at lower temperatures (261, 110, 208). At 55 C many of the thermophilic cells become long, slender, frequently curved, and show marked granulation; at 37 C, cells of the strains examined are smaller, more uniform in size, and stain homogeneously. Pleomorphic forms in cultures of thermophilic microörganisms have been described by various observers (23, 135, 234, 236). The microscopic appearance of the organisms cultivated at different temperatures, thus, has led many workers to believe that the real optimum temperature of the thermophilic forms is much lower than generally assumed.

The selection of the optimum temperature for a thermophilic form, of course, depends upon the criterion selected. Whether we select the region of maximum viable cell yield or maximum reproductive rate is unimportant, provided the latter is compatible with the preservation of the species. For any organism there will be a discrepancy between the temperature at which these two maxima occur, and it is apparent that this discrepancy will vary directly with the breadth of the over-all temperature range for growth. It has been the recent practice to use the point or range of highest reproductive rate in the designation of the optimum temperature for growth of thermophiles.

2. Growth at Low Temperatures. The thermophilic microörganisms, arbitrarily characterized by an optimum temperature above 50 C, exhibit considerable latitude in their over-all temperature range for growth. A large number of thermophilic bacteria have been found to grow at 37 C, and even at 20 C. An equally great number of cultures have a very high minimum temperature for growth. The fact, that the latter organisms were found in geographic regions where their minimum temperature for growth was seldom, if ever, reached, gave rise to a lengthy and heated argument with regard to the growth characteristics of the thermophilic microörganisms.

One school maintains that all organisms which grow at high temperatures are thermotolerant, i.e., thermophiles grow at high temperatures and also, more

slowly, at lower temperatures. Here adaptation to an elevated temperature range is never complete; and, many cases (2, 144, 251, 106) have been described to substantiate this belief. Evidence (234, 193, 182, 144) also has been presented to show that the environment (culture medium) exerts an important influence on the temperature limits for growth, and may be responsible for the inability of many workers to obtain growth at both ends of the temperature range. Although scattered evidence of this effect on thermophilic bacilli occurs in the literature of the last century, the first controlled experiments were conducted in 1911 by Koch and Hoffmann (144). They found that thermophilic bacilli isolated from the soil would not grow in artificial culture media at temperatures below 40 C, but grew well in soil at temperatures as low as 20 to 30 C. Thus, they derided Fischer's theory of dormancy of thermophiles (82) and Miehe's explanation that thermophiles in temperate regions required heated piles of organic matter for growth (171). Thermophilic bacilli, according to Koch and Hoffmann, proliferate at low temperatures when in their native environment. Noack (188) concedes such a possibility in the case of bacteria, but not of molds. The influence of the composition of an artificial culture medium on the temperature characteristics of thermophilic bacteria has been studied by other workers (182).

Rabinowitsch (215, 216) found that thermophilic bacteria that she isolated grew at 33 C if cultured anaerobically, whereas no growth was evident aerobically at this temperature. Thus she was led to conclude that oxygen tension was the factor which determined the minimum temperature. Her findings were confirmed, in part by Schütze (238) and Ambrož (2), but opposed by the results of Oprescu (193), Miehe (171), de Kruyff (150), and Shaw (243). Nègre has concluded from his studies that all obligate thermophiles are obligate aerobes and all facultative thermophiles are facultative aerobes. Recent studies (42) have revealed that there is considerable diversity with respect to the effects of temperature upon oxidation-reduction relations, which would indicate that the results of Rabinowitsch are limited in scope. Since the time of Rabinowitsch, thermophilic species have been found with oxidation-reduction potential requirements representative of aerobes, facultative anaerobes, and anaerobes (80, 38, 165, 166).

Morrison and Tanner (181, 182) have suggested that in the observation of growth of thermophilic bacteria at low temperatures, the time element is of greatest significance. They maintain that many investigators have not waited long enough for proliferation to become apparent, and have concluded incorrectly that the organisms were incapable of growth. Quantitative studies (106, 251) indicate that thermophilic bacteria multiply so slowly at low temperatures that an increase in number of cells has been overlooked consistently. Hansen (106) found a generation time of 370 minutes at 20 C for the thermophile he studied. Therefore, when dealing with thermophilic organisms, the ordinary method by which an inoculum is spread over an agar slant and observed for growth after a period of incubation, was considered inadequate. Others (89, 129) have proved that the apparent failure of proliferation in some cases is not the result of the insensitivity of the method.

It is apparent that this school had little difficulty in fitting their thermophiles into the economy of nature in temperate zones; and repeated emphasis has been placed upon the importance of their activity in the soil (3), surface waters (8), and the intestine (215).

The second school recognized two groups of thermophiles: one with a wide temperature range, including the usual mesophilic range; the other with a narrow range, the lower limit of which is usually above 30 C. These groups may be designated by the terms "eurithermal" and "stenothermal", respectively.

Although it was not until the past decade that a relatively clear picture of the latter group was presented (129), the literature contains numerous accounts which satisfy the definition of the stenothermal type. But we find also that we cannot select an arbitrary temperature as the minimum temperature for growth of all stenothermal organisms and thus differentiate these two types of thermophiles. An undeniable transition between the eurithermal and the stenothermal groups is indicated.

Many of the twenty species of MacFadyen and Blaxall (163) had minima between 60 and 65 C; Bergey's (19) *Bacillus thermodiasticus* and *Bacillus thermononliquefaciens*, 50 C; *Bacillus thermophilus vranjensis* of Georgevitch (93, 95), 49 C. A minimum of 45 C was found for the organisms of Hussong and Hammer (119), Donk (66), Gilbert (96), and Georgevitch (94); 42 C for *Bacillus pepo* of Shaw (243), and, 40 C for the species of Sames (234).

Many other organisms with a minimum temperature between 37 and 45 C have been described (150, 44, 258–260, 170, 79).

Bergey (19) has described organisms, such as *Bacillus thermoliquefaciens*, *Bacillus lobatus*, and *Bacillus thermotranslucens*, which show slight growth at 37 C. *Bacillus thermocellulolyticus* of Coolhaas (53) was found to have a minimum of 35–37 C. Schütze (238), Miehe (171), and Kedzior (139) have described thermophiles with temperature minima in the region of 30 C.

However, the clarity of this gradual transition does not remove the question of the existence of thermophiles with very elevated minimum temperatures. The suggestion that failure of proliferation below a critical temperature is more apparent than real, has been cited earlier. The time element in incubation of thermophiles at temperatures below their apparent minimum has been shown to be unimportant in the case of several typical strains of obligate (stenothermal) thermophiles studied by Cameron and Esty (38). Dextrose broth and corn juice inoculated with spores of thermophilic bacilli, with a minimum temperature of 42 to 45 C, showed no activity during five years of incubation at 35 to 37 C. Similar inoculations into canned corn, held at 22 C and 37 C, exhibited no activity during three years of incubation. All samples, however, when placed at 55 C invariably gave rise to rapid proliferation and acid production. Analogous results were obtained by Shaw (243), using *Bacillus pepo*, in studies of shorter duration. Recent work by Curran and Evans (56) indicates the necessity for preliminary heat-shocking of spores of thermophilic aerobes before germination will proceed. Additional observations of the rapid loss in vitality of heat-activated spores, when subjected to an unfavorable environment, (57) provides a possible explanation of the frequent notation that thermophilic cultures become sterile when stored at room temperature.

The early literature on the survival of thermophilic microörganisms at subminimal temperatures contains many inconsistent statements. Thus, Tsiklinsky (359) found that her spore-free cultures of *Thermoactinomyces vulgaris* survived storage at a subminimal temperature, while Miehe (171) observed the rapid death of spores of *Actinomyces thermophilus* in artificial culture media. Similar opposing results have been presented for spore-free cultures of thermophilic bacilli at temperatures below their apparent minimum (171, 234). Noack (188) investigated the effect of subminimal temperature on the vegetative cells and spores of five thermophilic molds, a thermophilic actinomycete, and a thermophilic bacillus. The vegetative forms of the three molds and the bacterial species exhibited very low resistance to a temperature slightly below the minimum, while the other two mold species and the actinomycete showed a very low death rate under the same conditions. Spores of all forms, however, were highly resistant. The susceptibility to the effects of low temperature bore no relation to the minimum temperature for growth of the organism in question, to the temperature of incubation prior to storage at a subminimal temperature, or to the composition of the culture medium. Death at such temperatures was explained by the assumption of a unique respiratory system, as suggested earlier by Miehe (171), or a membrane with a very critical response to decrease in temperature.

Shaw (243) has another explanation of the observed effect of low temperature on thermophilic bacteria. Her cultures, when stored for a prolonged time at room temperature failed to yield viable inocula. Subsequent study revealed that upon storage, turbidity disappeared from tubed liquid media and samples taken from the upper portion of the media proved to be sterile in many cases, while samples removed from the bottom portion yielded viable spores. The tendency of the culture to sporulate and of the spores to settle out permitted a number of conclusions.

The occurrence of bacteriophage active against thermophilic bacilli (1, 145) has suggested phage as possible growth inhibitor, effective at lower temperatures. Abundance of growth at elevated temperatures could be explained by the heat-lability of the phage in question. Partial inactivation of the phage at the minimum temperature for growth of the organism would account for the observed effect. However, phage with these unusual properties has not been detected in cultures of thermophilic bacteria.

Quantitative population studies undertaken by Imsenecki and Solnzeva (129) have established that workers, who have denied the existence of bacteria of the stenothermal type and have claimed that all thermophiles proliferate at low temperatures, have not had the opportunity of examining members of the stenothermal group. Gaughran (89), in a study of five stenothermophilic bacteria has demonstrated that the environmental factors, such as, nutrient and nutrilite supply, inhibitors, oxygen and carbon dioxide supply, oxidation-reduction potential of the medium, relative hydration and pH of the medium, exert a considerable influence on the growth response of these organisms within the temperature range of approximately 38 to 75 C. Proliferation did not occur

under any combination of conditions at temperatures below 38 C. Manometric data, used as a measure of growth, in all cases paralleled population data. The behavior of the stenothermophilic bacteria suggests peculiarity in their enzyme complement or, perhaps, a unique structural chemistry.

Analyses of the minimum temperature for growth have been predicated more on hypothesis than on experimental data. Ideal material for the study of this fundamental problem is found in the inability of the stenothermal thermophiles to metabolize and reproduce at temperatures which are suitable for most other forms of life. Generally, attempts at an explanation have not gone beyond the suggestion that these organisms possess a unique mechanism, which has apparently replaced the mesophilic mechanism lost during the adaptation process. One study (71) has indicated that the respiratory enzymes of thermophilic forms do not function at ordinary temperatures; others (111, 152) suggest deficiencies in the respiratory and hydrolytic systems.

Growth processes and processes furnishing energy are usually placed in separate categories and their interdependence frequently minimized. Although both processes are conceded to be enzyme-catalyzed, the temperature range of the energy-yielding processes is much wider than that of growth processes. Reactions involving energy liberation and syntheses are so interlinked that the retardation of any single reaction might prevent completely the functioning of others and thus make growth impossible. The absence of growth and proliferation in stenothermophilic cultures may thus be related to the failure of one or more steps in this metastable chain of exothermal and synthetic reactions. Synthetic reactions in biological systems are still obscure and our knowledge is based largely upon the isolated endproducts and a few intermediate compounds. The respiratory mechanism, including all chemical processes by which energy is made available to the cell, has been studied extensively in some animals and plants. Investigation of the activity of the bacterial respiratory enzymes with respect to temperature has been confined largely to organisms with relatively low minimum growth temperatures. References made by several workers to the inactivation of bacterial enzymes by low temperatures and to deficiencies, both qualitative and quantitative, in the respiratory mechanism of thermophiles have no supporting experimental data.

Foter and Rahn (85) in an analysis of minimum temperature state that the most common explanation of cessation of growth at low temperatures is the assumption that the numerous interlinked reactions of the cell are influenced differently by a change in temperature, with the result that the growth mechanism is upset. The accumulation of toxic metabolic products within the cell is not considered a possible cause for a disturbance of the growth mechanism in the case of bacteria or other cells with large surface area. Excessive viscosity of the protoplasm is also discounted. The change in permeability induced by changes in temperature is cited as a possible explanation and its relation to the consistency of lipids suggested. In an accompanying laboratory study they demonstrated that lactose fermentation by several lactic acid organisms takes place at a temperature below the apparent minimum temperature for growth.

A more recent study of the stenothermal *Bacillus cellulosae-dissolvens* (129) indicates that this situation is not encountered invariably. Flasks containing cellulose were inoculated with the organism and incubated at a high temperature until fermentation was well advanced. Then a sample was removed, and the flasks placed at 20 C for ten days. No change in quantity of the hydrolytic products of cellulose or volatile acids could be detected. However, all the extracellular hydrolytic enzyme preparations from thermophilic bacteria which have been examined to date exhibit activity at ordinary temperatures (91).

In a recent kinetic study of the effect of temperature on the respiratory mechanism of the stenothermophilic bacteria (91), the respiratory mechanism and its various enzymic components were found to function at temperatures far below the minimum for growth. In every case the rates of the individual reactions involved in the respiratory chain increased exponentially with temperature up to the temperature at which inactivation became apparent. Identical energies of activation for the over-all respiratory system and its enzymic components were obtained at temperatures above and below the minimum temperature for growth of the organisms. This observation is significant in the indication that there is no fundamental difference in the effect of temperature on the respiratory systems of stenothermophilic and mesophilic bacteria. The similarity in nature of the enzymes functioning in the respiration of mesophiles and thermophiles also is suggested.

The stenothermal thermophiles have been used to test the general hypothesis of Heilbrunn (112) and Bělehrádek (15) which related heat resistance of an organism and the melting point or degree of saturation of its protoplasmic lipids (90). The results of this study suggest a possible extension of this hypothesis, namely, that the temperature range for growth is a function of the degree of saturation of the cellular lipids. A large proportion of the cellular lipids of the stenothermophilic bacilli was found to exhibit a high degree of saturation over the entire temperature range for growth. Thus, as the minimum temperature for growth is reached a large proportion of the lipids approach solidity. The incompatibility of this situation with active metabolism at lower temperatures has been pointed out, as well as the inference that the consistency of the fats elaborated by the stenothermophilic group of bacilli may prevent active metabolism at low temperatures and fix the minimum temperature for growth.

TEMPERATURE ADAPTATION OF MICROÖRGANISMS

The assumption that thermophilic forms were the result of an adaptation process stimulated short-term adaptation experiments employing both plants and animals. On the suggestion of Darwin, Dallinger (60) studied three flagellates and cited the results in his presidential address before the Royal Microscopical Society in 1887. These protozoa, which originally grew at about 16 C and had their maximum at 23 C, by gradual exposure to increasing temperature over a period of seven years, were made to grow normally at 70 C. Davenport and Castle (62), by incubating frog eggs at temperatures of 10 C above normal, obtained tadpoles with a temperature tolerance 3° above normal.

Signs of adaptive changes have also been found in Fundulus, insects, coelenterates, protozoa, human erythrocytes, and isolated frog nerve (16).

Dieudonné (64, 65) observed an elevation in the growth temperature of *Pseudomonas fluorescens* and of *Bacillus anthracis* during his study of the behavior of various bacteria to unfavorably high temperatures. Similar response, although very slight, has been reported for other bacteria (260, 233, 203, 183, 167) and molds (256). Attempts at an adaptation, over a period of a year, of ten spore-forming mesophilic bacteria by Casman and Rettger (42) were unsuccessful. Desiccation, and growth in concentrated solutions of sucrose, peptone, or sodium chloride also failed to increase heat tolerance (42, 72). "Temperature shocking", a selective process, has been successful in shifting the maximum growth temperature of two non-sporeforming bacteria several degrees (32).

Jancke (135), noting a similarity between the thermophilic organisms and the *Bacillus mesentericus* group (fuscus, ruber, vulgatus, panis viscosi), attempted to develop heat resistant strains from the mesophilic species, as well as to adapt heat resistant strains to lower temperatures. Unfortunately, all the "thermophiles" which he developed, and all except one which he isolated, would not survive cultivation at 60 C for more than two or four transfers. In addition, at this temperature they lost their ability to produce spores and coagulate and peptonize milk. They were indeed similar in character to species of the "mesentericus group" cultivated at 55 to 60 C, but they failed to fulfill the author's definition of a "thermophile". One organism which he isolated and designated as an obligate thermophile grew optimally at 60 C for many transfers and never yielded a strain which would grow below 40 C. Jancke was attempting to provide data for the theory of Lieske (159) which maintained that a radical or sudden change in temperature was capable of spontaneously inciting a mutation, thus adapting the organism to the new environment. Both Jancke and Lieske, however, admit that the spontaneity of this "mutation" may be more apparent than real.

An appreciable increase in temperature tolerance appears to require a great length of time, and the sudden development of thermophilic forms as suggested by Lieske (159) and Kluyver and Baars (143), frequently has been considered as very doubtful (129). Kluyver and Baars offer the interesting implication that many thermophilic cultures are physiological artefacts. This is based upon a study of *Vibrio thermodesulfuricans*, supposedly derived from *Vibrio desulfuricans*. The thermophilic organism is strictly anaerobic and does not form spores. Minute amounts of oxygen sterilize the culture as the temperature of cultivation approaches the minimum and the rate of metabolism falls off. The organism is considered to be a physiological artefact, because it is obviously unsuited to occur in nature.

Starkey (248) has stressed the significance of the results of Kluyver and his students from their studies of the effect of environment on the characteristics of microörganisms. The effect of temperature on the morphology of the vibrios and spirals, and the ease with which adaptation occurs in these forms cannot

be minimized. Further clarification of these observations was found in the course of an investigation of a sporogenous vibrio, *Sporovibrio desulfuricans* Beijerinck (247, 248). The very wide temperature range for growth of the vibrios and spirals (*ca.* 40 C) suggests that other bacteria can be made to grow at temperatures well above the generally accepted maximum temperature by a gradual adaptation process. Thus, by gradually altering the temperature of incubation, the cardinal temperature characteristics for growth (i.e., minimum, optimum, and maximum temperatures) of a particular organism can be changed. Such a general situation would detract from the significance of these terms.

The "lipoid liberation theory" of Bělehrádek (15), which links the heat "adaptation" of the protoplasmic fats with the adaptability of the whole organism to high temperatures, has been substantiated in part by the work of Fraenkel and Hopf (86). The results of their work led to the suggestion that although the physical nature of the lipids may have a decided influence on the chain of physiological processes, the theory is entirely inadequate in explaining the phenomena of heat injury and heat adaptation.

Explanations of thermobiosis based on the assumption that thermophilic organisms possess a unique mechanism, which apparently replaces the mesophilic mechanism lost during the adaptation process, has led to a further assumption of enzyme adaptation. Although the question of temperature adaptation of biological "ferments" was under consideration (81) long before the term "enzyme" had been proposed, sight of the basic problem has been lost in later years. Some workers (142) have been led to believe that entirely different enzymes are concerned in the metabolism of cold- and warm-blooded forms, although it has frequently been reported (148) that the enzymes of cold-blooded animals at lower temperatures are as active as the enzymes of warm-blooded animals at higher temperatures. The classical example of a typical enzyme adaptation, presented by Kjeldahl (141) in 1881 for invertase of top and bottom yeast, was accepted for thirty years (192), until disproved by von Euler and his students (272, 273). Harder (109) has claimed indirect evidence for the adaptation of assimilatory enzymes of one of the higher plants to the temperature of the environment; one of his students (197, 162), however, upon extending this work to the diastase of *Aspergillus niger* and *Penicillium glaucum* in a poorly controlled experiment, concluded that there was no adaptation of enzymes to change in temperature at which these enzymes were formed.

When we realize that plant enzymes in general exert their optimum activity between 50 and 60 C (260), although they are seldom subjected to such temperatures in nature, the optimum growth of bacteria at 50 and 60 C, and continued growth at even higher temperatures is understandable to some degree. However, among the thermophiles it is true that the few enzymes which have been studied in growing cultures and resting cell preparations apparently have slightly higher optimum and maximum temperatures than mesophilic forms (91, 130, 284). A study of the activity of an extracellular hydrolytic enzyme in a growing culture, where the replacement of inactive enzyme molecules is an important factor, cannot reveal, however, the temperature characteristics

of the enzymes in question. Although there is no conclusive evidence that the enzymes of thermophiles have higher optimum and maximum temperatures than corresponding enzymes of mesophiles, the similarity of response of the respiratory enzymes of these two groups of organisms to change in temperature has been demonstrated (89). There is also no direct evidence that the enzymes of these organisms, or of any other organisms, have arisen as the result of adaptation of enzymes to temperature.

Attempts at lowering the cardinal temperature characteristics of an organism have been made, but these studies provide very little dependable data. Repeated freezing and thawing was ineffective in lowering the minimum temperature for growth of the cholera and typhoid bacteria (25). Similar failures, by gradual decrease in the incubation temperature over a long period of time, also have been reported in efforts to depress the optimum temperature of thermophilic bacilli (99), and the minimum temperature of *Bacillus anthracis* (64), of a thermophilic actinomycete (96), and of a thermophilic mold (188). Loss of the ability to grow at high temperatures upon prolonged cultivation in their lower temperature range (161), as well as the loss and subsequent recovery of thermal resistance (70), has been reported for a number of thermophilic microorgansims. A small number of such observations have been taken as evidence for the instability of the thermophilic species; and the reversibility of the adaptation of thermophiles to temperature is assumed commonly (99).

That some adaptations to environmental conditions occur fortuitously by mutation cannot be denied. The probability, however, of observing such a change by prolonged cultivation of an organism under a slightly modified environmental condition is hardly to be expected. Attempts at accommodation by gradual change of temperature in an effort to duplicate the process of natural selection are also unjustified, as attested by the very questionable success in the work undertaken in the past. The behavior of the vibrios in their ease of adaptation to changes in temperature must be considered unique, on the basis of our present knowledge.

SUMMARY. IMPORTANCE OF THERMOPHILIC MICROÖRGANISMS

The preceding examination of our accumulated knowledge of the thermophilic microörganisms suggests that the paradox in their behavior is more apparent than real. The similarity in the physiology of mesophilic and thermophilic bacteria is evident. Differences are to be found only in the intensity with which biochemical changes are effected. The gradual and imperceptible transition from the thermophile to the mesophile also suggests that the thermophiles do not represent an isolated biological group. As such, they constitute very significant material for study in order to augment our present very meager knowledge of the mechanisms involved in thermobiosis. Organisms of the group which exhibit an elevated minimum temperature for growth offer excellent material for clarifying the temperature responses of the growth processes. Although the behavior of this type of thermophile in pure culture is incompletely explained, a study of the activities of these organisms in nature may provide an

important contribution to our knowledge of symbiotic relationships among microörganisms. It has been the aim of this review to present a complete picture of the scattered knowledge of the thermophiles against the background of a vast number of problems which they present, as well as to emphasize the importance of these organisms for study.

Today thermophilic microörganisms come to our attention largely because they are responsible on occasion for the spoilage of processed foods. Although many thermophiles were isolated from canned food early in the history of the canning industry, attention to these organisms as the cause of spoilage began with the work of Barlow in 1912 (9). Since that time the number of descriptions of isolation of the organisms and the types of spoilage has become so great as to make even a tabulation prohibitive. The subject has been discussed and reviewed on many occasions (165, 249). Particular attention has been given to the presence of thermophilic bacteria in the various ingredients, such as starch and sugar, entering into the manufacture of foods (35–37, 43, 50, 249). The thermophilic bacteria were of great importance during the war in the case of certain canned foods designated as "commercially sterile", which rapidly spoiled upon storage in the tropics (10).

Rather recent recognition was made of thermophilic bacteria in milk, as a result of the sporadic appearance of large numbers of so-called pin-point colonies in the plate counts of pasteurized milk. Since these organisms proliferate during the pasteurization process, a sample of milk may have a higher bacterial content after pasteurization. The significance of these bacteria lies in their ability to ferment lactose, or less commonly decompose proteins, and cause undesirable flavors or odors. Thermophiles are responsible to a large extent for pin-point colonies, although it has been shown (209) that other types of bacteria are also involved. Many health authorities are inclined to disregard heat resistant organisms in milk because they are non-pathogenic; others maintain that the number of thermophilic and heat resistant bacteria constitute a good index of undesirable conditions in the production of milk and that the organisms can be controlled by the observance of rules of cleanliness. Since the standard plate count does not reveal all thermophilic bacteria present in a sample, and therefore does not present an accurate picture of the bacterial condition of the milk, other routine methods of examination have been proposed (249).

In addition to the undesirable activities of the thermophiles, the beneficial aspects of these organisms also must be noted. They have been considered earlier in the discussion as potential agents in the controlled fermentation of cellulose to useful products. The versatility of the intense biochemical activity of thermophilic microörganisms, taken as a group, offers many opportunities for their industrial application. Thermophiles have found application in the recovery of vegetable oils and fats (12) and in the degumming of silk (138). The high metabolic rate of thermophiles which results in rapid accumulation of a large quantity of extracellular enzymes in the medium is also a situation rife with possibilities. Amylase (125) and "degummase" (128) of thermophilic bacilli have found industrial use as enzyme preparations.

It is obvious that the problems of thermobiosis are not purely academic; nor are they of interest to industry merely because of the presence of thermophilic bacteria in foods and products. Thermobiosis may perhaps also provide a tool with which desired biochemical changes may be effected more rapidly.

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